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Nucleotide Oligomers

J. Randall Good
Aileen Huang-Saad

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PREFACE

The foci of the Advanced Technology in Molecular Biology task include identifying shortcomings in current technology and areas where DARPA may be able to assist in driving further development in this area. In essence, the objective is to identify areas in which progress has stalled and where short-term involvement may jump-start the progress in this area.

One such area is DNA computing, that is, using the short-chain DNA sequences, nucleotide oligomers, to complete mathematical calculations. It has been suggested that a key issue in this area is to miniaturize in vitro synthesis of these oligomers. We have also been asked to investigate other areas of biomedical science that would benefit from this development.

The purpose of this paper is to describe areas that are currently using nucleotide oligomers technology and those that might benefit from an improvement on the current synthesis techniques. Current techniques generally address the large-scale synthesis of single sequence polymer. The improvement of techniques that address the synthesis of many different sequences, each in small number, as would be required for DNA computing, will assist in the development of the molecular techniques described.

CONTENTS

A. PURPOSE OF THIS PAPER 1

B. GENERAL BACKGROUND 1

C. RIBONUCLEIC ACID (RNA) 4

D. ROLES OF NUCLEIC ACIDS IN BIOMEDICAL RESEARCH 5

1. DNA Synthesis 5
2. Hybridization Probes 7

E. EMERGING MOLECULAR TECHNOLOGIES INVOLVING OLIGONUCLEOTIDES 8

1. Antisense 8
2. DNA Triplex 8
3. Ribozyme 9

F. FORWARD-LOOKING DEVELOPMENTS 10

G. FUTURE ROLES OF NUCLEIC ACID TECHNOLOGIES 11

H. CONCLUSIONS 12

Glossary GL-1

References Ref-1

FIGURES

1. Each of the Five Nucleotide Monomers Used in Nucleic Acids Synthesis 2

2. (a) The Carbons of the Ribose Sugar are Numbered 1´–5´ 3

   (b) Once connected, the Chain Has an Exposed 5´ Sugar on One End of the Molecule and an Exposed 3´ Carbon on the Other 3

3. Several Overlapping Oligonucleotides Can be Synthesized and "Pieced Together" by Allowing Them to Hybridize and Therefore Create Larger DNA Molecules 6

NUCLEOTIDE OLIGOMERS

A. PURPOSE OF THIS PAPER

The purpose of this paper is to describe the chemical and biological basis of nucleotide oligomers (oligonucleotides) and nucleic acids. This paper will also describe (1) several uses of these molecules as tools in laboratory and clinical medical research and (2) several emerging technologies that exploit the increasing knowledge of molecular biochemistry of nucleic acids.

This paper will address the roles of nucleic acids in biological research and some emerging technologies involving these molecules. Many of the experimental techniques involve the use of oligonucleotides. Oligonucleotides are chains of nucleotides that can range in size from a few bases to a few hundred bases. Oligonucleotides are single stranded unless otherwise noted.

B. GENERAL BACKGROUND

Deoxyribonucleic acid (DNA) is commonly known as the repository of genetic information. That is, the information within the DNA sequence of an organism's genome gives rise to proteins which ultimately result in the physical traits, visible or not, of that organism. Less widely known is ribonucleic acid (RNA), which acts as an intermediate messenger between the database (DNA) and the machinery (protein). In addition, in some viruses (*retroviridae*), RNA in the genome serves as the final repository for the genetic information (for complete nucleic acid review see Reference 1).

DNA and RNA are members of a class of biological macromolecules known as nucleic acids. A nucleic acid is a macromolecule composed of a linear array of nucleotides joined by phosphodiester bonds. Nucleotides comprise three components: a five-carbon sugar (ribose), a nitrogenous base, and an inorganic phosphate group (Figure 1). The sugar and phosphate are invariable among each monomeric subunit. It is the nitrogenous base, however, that determines whether the nucleotide is adenosine (A), guanosine (G), cytidine (C), or thymidine (T), the "letters" of the DNA alphabet.

The ribose sugar comprises five carbons. Each carbon is numbered 1´–5´ for identification (Figure 2a). The nucleotide chain of a nucleic acid strand is constructed by

linking the phosphate group attached to the 5´ carbon of one molecule to the 3´ sugar of the next. This results in a sugar phosphate backbone that is often described directionally as 5´ to 3´, representing an exposed 5´ carbon on one end of the chain and an exposed 3´ carbon on the other (Figure 2b). Much like the English language is written and read from left to right, so is DNA 5´ to 3´. When referring to various features in a DNA strand, the 5´-most feature is upstream of the 3´ feature. A gene within the DNA sequence is "read" in this 5´ to 3´ direction.

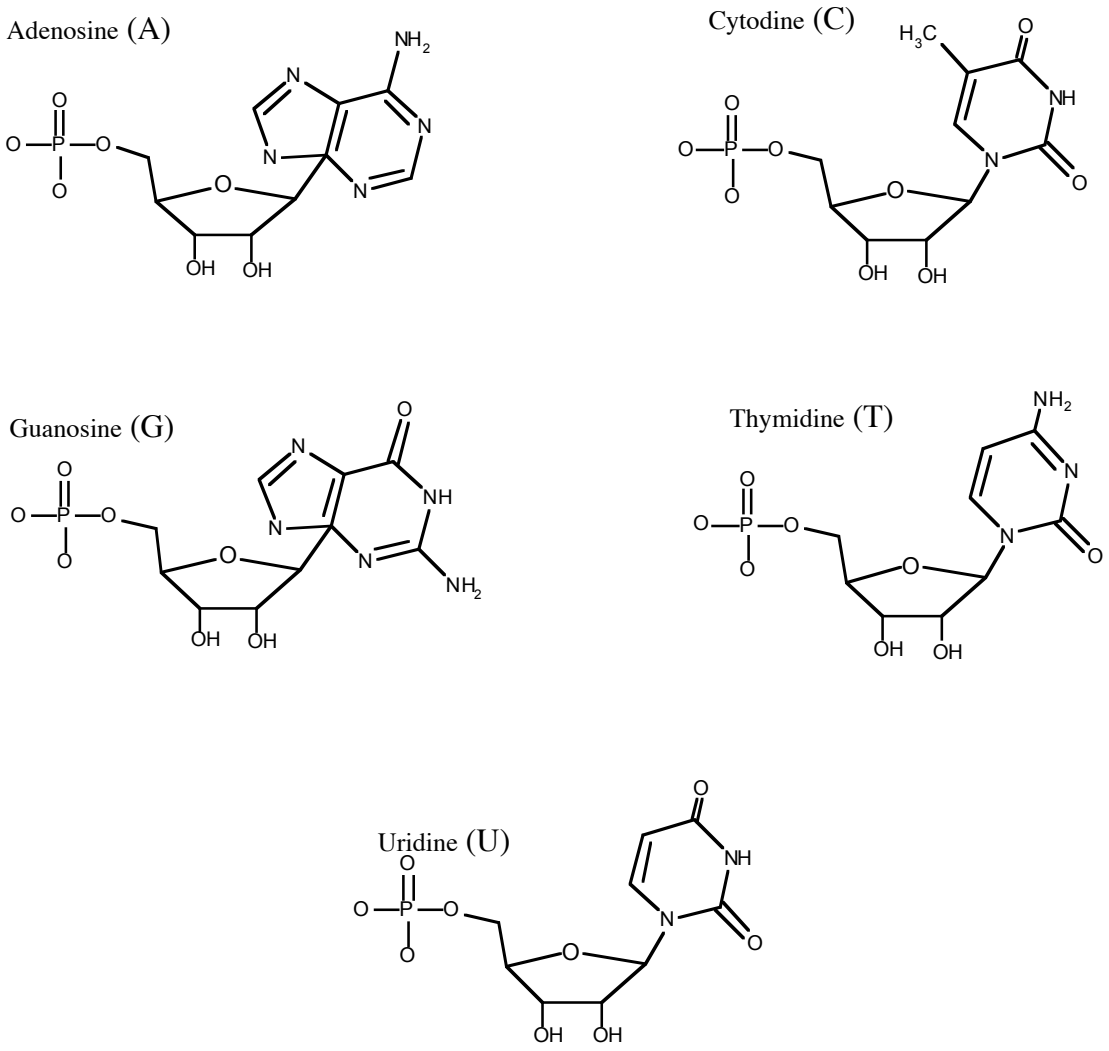
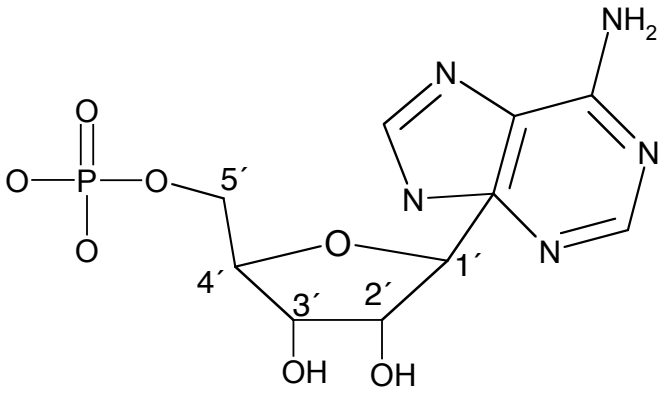
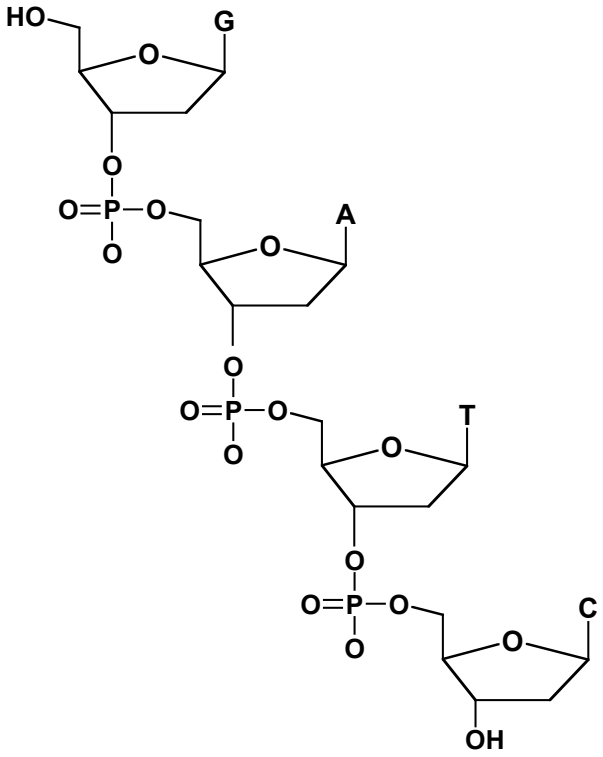


Figure 1. Each of the Five Nucleotide Monomers Used in Nucleic Acids Synthesis. The top four (A, C, G, T) are found in DNA. In RNA uridine takes the place of thymidine. These nucleotides are shown in their monophosphate form. Prior to integration into the nucleic acid chain, the monomers are present as triphosphates. During synthesis, they are converted into mono phosphates and connected through 3´ to 5´phosphodiester linkage (as described in detail in the text).



(a)



(b)

Figure 2. (a) The Carbons of the Ribose Sugar Are Numbered 1´–5´. The variation deoxyribose (in DNA) is the absence of a hydroxyl group (OH) on the 2´ carbon. Nitrogenous bases are attached to the 1´ carbon, and the nucleic acid backbone links the sugars with a phosphate group that attaches between the 3´carbon on one sugar and the 5´ carbon on another.
(b) Once Connected, the Chain Has an Exposed 5´ Sugar on One End of The Molecule and an Exposed 3´ Carbon on the Other.

Compositionally, the differences between DNA and RNA are few in number. First, in an RNA sequence, uridine bases are substituted for thymidine. Second, the sugar residue is different between the two molecules. DNA uses a modified version of the ribose sugar known as 2´-deoxyribose. That is, the carbon at the 2´ position is lacking the hydroxyl (OH) group (Figure 2). It is this minor difference that is responsible for many of the chemical differences between DNA and RNA and the increased reactivity of the RNA molecule.

Another difference between the two types of nucleic acids is that RNA largely exists as a single strand, whereas DNA is found as a double-stranded molecule. There are, of course, exceptions to both of these cases, some of which will be discussed later. The double-stranded configuration of DNA decreases the molecule's chemical reactivity and hence increases its stability. The two strands of DNA combine in an antiparallel manner. That is to say that the 5´-end of one chain aligns with the 3´-end of the other and the strands run in opposite 5´ to 3´ directions. The two strands are not attached to each other but, rather, they interact electrostatically. The G residues on one strand align with the C residues on the other. Likewise, A residues form base pairs with T residues.

It is these electrostatic interactions that hold the double stranded DNA together. Hybridization is the term used to describe the coming together of two single DNA strands by the base pairing of G ≡ C and A = T residues. It should also be noted that G ≡ C interactions are stronger than those formed by the A = T base pair. Thus, double-stranded DNA molecules with a greater GC content are more stable than those that are rich in A and T residues. For our purposes here, references to DNA imply double-stranded DNA molecules unless otherwise noted.

C. RIBONUCLEIC ACID (RNA)

A gene is a region of DNA that contains all the information needed to give rise to one protein. This information includes the regulatory elements that control when a gene is turned on, the sequence or description of how to assemble the protein, and information on where to put the finished product. In some organisms, genes can be found to contain introns and exons. In order to make a protein, the intron sequences will be removed and the remaining exonic sequences will give rise to the protein. Because the strands of double-stranded DNA are complementary and not identical, only one of the strands, called the sense strand, contains the genetic coding information. The other, complementary, strand is referred to as the noncoding strand and is said to have anti-sense sequence.

When a gene is expressed, the cellular machinery transcribes the gene, making an RNA copy. This sequence is identical to the coding sequence of the DNA in the gene, except the thymidine residues are replaced by uridine. This RNA is then processed, or spliced, to remove intronic sequences. This splicing pieces together the exons, the regions of RNA that encode the gene product. The RNA, now called a mature or messenger RNA (mRNA), gives rise to a protein through a process called translation.

D. ROLES OF NUCLEIC ACIDS IN BIOMEDICAL RESEARCH

Oligonucleotides can be easily purchased from a range of retail biological supply companies in the United States and worldwide. Over the past decade, these molecules have become quite inexpensive, and the products can be obtained rapidly (less than 24 hours). Oligonucleotides have been used in molecular biology research for some time. These molecules are most commonly used in DNA synthesis reactions and as hybridization probes. Many variations of these two molecular reactions have been used experimentally; several will be discussed.

1. DNA Synthesis

DNA synthesis is a complex reaction involving a number of enzymes for its initiation. The actual synthesis, however, is performed by single enzyme and four simple monomeric subunits. The enzyme responsible for DNA synthesis, DNA polymerase, cannot synthesize DNA de novo. That is, DNA polymerase can add nucleotides to a growing chain, but it requires a short chain, or primer, that it can extend. In vivo, this is accomplished by another enzyme, often called a primase, which synthesizes a short RNA primer because RNA can be synthesized de novo (i.e., transcription). Starting from this primer, DNA polymerase then extends the short stretch of RNA, resulting in the new DNA strand. However, using this technique in vitro would mean adding another enzyme to the reaction, which also means more variables and a more complicated procedure. Instead, a short oligonucleotide primer is often synthesized separately and added to the reaction. Two common synthesis reactions are DNA sequencing and polymerase chain reaction (PCR).

During a DNA sequencing reaction, a new strand of DNA is synthesized utilizing specific marker molecules in place of some, or all, of the nucleotide subunits. These markers can be recognized as G, A, T, or C and are used to identify the sequence of the DNA strand. A complete biochemical description is beyond the scope of this paper, but what is important here is that this synthesis requires a primer for the polymerase enzyme

to synthesize this new strand of DNA. The primer is designed to hybridize to the existing DNA template strand upstream of the region to be sequenced. The DNA polymerase will then synthesize the new strand of DNA that corresponds to the desired region. From this new strand containing the marker molecules the sequence of the DNA can be read.

Similarly, PCR is a modified DNA synthesis reaction. PCR is a technique for amplifying DNA by synthesizing numerous identical copies in iterative rounds of DNA synthesis. PCR, like a sequencing reaction, uses a DNA polymerizing enzyme that requires an oligonucleotide primer in order to begin the synthesis. Unlike sequencing reactions, PCR requires that two DNA primers be added to the reaction. These primers flank the region to be amplified so that both strands of DNA are synthesized in multiple rounds of synthesis. This results in numerous copies of double stranded DNA.

The primers used for PCR are generally longer than those used for sequencing. Longer primers mean greater hybridization energy, an increase in stability of the double stranded molecule, and an increase in the likelihood that the primer will hybridize (bind) to the correct region of the DNA. PCR primers can also be designed to contain a non-native DNA sequence, that is, a sequence that does not already exist in the template DNA. By using primers with an additional sequence, the newly synthesized DNA copies will also contain this additional sequence. The use of PCR in mutation and manipulation (changing or adding a sequence) will not be covered here.

In addition to their use as primers for DNA synthesis, oligonucleotides are also used for the de novo construction of large segments of DNA. Synthetic double stranded DNA (complementary oligonucleotides) can be used as linkers between other pieces of DNA, as spacers, or as a means of inserting foreign DNA into native sequence. On a larger scale, entire genes can be constructed by synthesizing overlapping, complementary oligonucleotides that represent the entire gene and allowing them to hybridize (Figure 3). This is sometimes necessary when the native genes are not readily available in a useful form or if a particular sequence modification is desired. Longer oligonucleotides can also be designed to make a complete double stranded DNA molecule. This technique does not get a great deal of use because in practice there are easier methods for obtaining large DNA molecules. These methods will not be discussed.

Figure 3. Several Overlapping Oligonucleotides Can Be Synthesized and "Pieced Together" by Allowing Them to Hybridize and Therefore Create Larger DNA Molecules

2. Hybridization Probes

DNA oligomers are also used as hybridization probes. The probing of nucleic acids by other nucleic acids is useful when trying to identify or isolate a specific DNA fragment in a large background or in a pool of undesired DNA fragments. The rational is that unknown DNA fragments can be treated so that they exist in a single stranded form. These DNA fragments are then mixed with a labeled (e.g., radioactive or fluorescent), single stranded DNA probe. The two strands will hybridize to become double stranded and the unknown fragment will now carry the radioactivity or fluorescence associated with the probe DNA.

This technique finds widespread use when the unknown DNA fragments can be spread out and fixed to a substrate such as paper or suspended in a gel. When the probe hybridizes to the proper DNA fragment, the desired fragment can be visualized and isolated from the unwanted background.

A more recent development using hybridization techniques involves the use of DNA microarrays, or chips. Microarrays are chip-based collections of DNA oligomers. In this case, however, the sequences of the DNA fragments on the chips are known and the unknown DNA is passed over them and allowed to hybridize. Using this process it is possible to identify what DNA is present in a pool of DNA.

Hybridization chips have become quite useful in gene expression analysis. For example, microarrays that contain all the genes that are commonly expressed by cancerous cells can be developed. By isolating the mRNA from a cell and allowing it to hybridize to the arrayed DNA, it is possible to determine if the gene expression pattern is similar to that of a cancer cell. A particular pattern of gene expression is often indicative of a cancerous cell.

This technique is also useful in developmental studies. Developing cells will express different genes at different stages of development. Using chips containing genes from various stages of development, a researcher can determine the cell's developmental progress.

Microarray and hybridization technology may be a useful tool in DNA sequencing (Ref. 2). That is, using a microarray containing all of the possible octomer sequences, hybridizing a DNA fragment of unknown sequence will give a specific hybridization pattern. Then, using a computer algorithm to analyze overlapping regions, the sequence of the unknown fragment can be pieced together. This protocol has some practical complication for blind sequencing, but has found practical use in identifying

single nucleotide polymorphisms (SNPs), DNA sequences that differ at only one base position among thousands. In essence, this technique can be useful in determining the presence of a genetic mutation (e.g., a disease gene).

E. EMERGING MOLECULAR TECHNOLOGIES INVOLVING OLIGONUCLEOTIDES

Nucleic acids are attracting a great deal of attention in the laboratory and in medicine for their potential as a research tool and a clinical treatment of disease. We describe three techniques that are currently being developed for such purposes. The first two are based on hybridization properties of nucleic acids; the final example is a more complex problem that takes into account the three-dimensional structure of the molecule.

1. Antisense

Antisense is a method used to block the expression of a gene by adding an oligonucleotide to the cell. The use of nucleic acids in antisense technology has evolved from an experimental tool in lab research to a potential treatment in clinical medicine. When a gene is expressed, DNA is transcribed into a single stranded RNA, which is an exact copy of the coding strand of DNA. This RNA, following post-transcriptional modification (i.e., splicing), will be translated into a protein sequence. A synthetic antisense RNA that is complementary to the mRNA will hybridize to the mRNA in vivo, rendering it double stranded an unable to be translated. Thus halting expression of the gene.

The gene is still expressed at the transcriptional level, but no protein is present. Because many cancers are based on the inappropriate gene expression, a synthetic approach to gene "silencing" is an ideal approach to its treatment.

2. DNA Triplex

Another method that can be used to "silence" the expression of a gene is called DNA triplex formation. DNA oligonucleotides can be designed to bind to the already double-stranded DNA, resulting in a DNA triplex. The formation and stability of a DNA triplex is dependent on several factors, including the DNA sequence, GC content of the triplex forming oligo (TFO), and other reaction conditions, the technical details of which will not be discussed.

Like the antisense technology, several biologically and chemically reactive moieties can be attached to the TFOs. Triplex formation can be very specific, depending

on the sequence and length of the TFO. This is a way to target a specific chemical reaction to a site within the genome.

DNA triplex technology is not as far advanced as antisense methods, but its potential is greater. Although both methods are designed to ultimately eliminate the presence of unwanted proteins in the cell, DNA triplexes can be formed to stop the transcription of the gene. Thus giving rise to no RNA. Additionally, TFOs can be created that will hybridize to a specific gene in the genome and damage the DNA at that specific site. This, in turn, will initiate the DNA repair mechanisms within the cell and can, through a process that cannot be completely described here, correct a mutant form of the gene.

Modifications of this repression system include conjugation of biologically active moieties to the antisense oligonucleotide (Refs. 3, 4). These molecules are often chemically active and can play a role in the premature degradation of the mRNA or the cross-linking of the RNA chain so that its inability to be translated is ensured.

For example, autosomal dominant retinitis pigmentosa (ADRP) is a genetic disorder that results in the degeneration of night and peripheral vision. The genetic defect lies in one, or both copies of a gene required for normal retinal structure and vision, rhodopsin. Triplex technology can be used to (1) block expression of the mutated form and allowing the correct form to be expressed alone or (2) initiate the damage and repair of the mutated gene, which can result in the correct sequence replacing the mutant form. This technology is progressing at a relatively slow rate because, in part, of the insufficient number of researchers and funding in the field.

3. Ribozyme

In addition to their roles as information repository and messenger, respectively, DNA and RNA also play structural roles in living cells. Because RNA is single stranded, it takes on complex secondary and tertiary structures. The three-dimensional structures of these RNAs is often likened to those of proteins and can be found as structural components of protein complexes like ribosomes (the cellular machinery for translating mRNA sequence into proteins), reaction catalysts involved in mRNA splicing, and stand-alone RNA enzymes known as ribozymes. In vitro experimentation has also produced nucleic acid enzymes from DNA.

Although proteins are undoubted responsible for catalyzing the vast majority of reactions within living cells, it has become apparent that the RNA catalysts are not only

required, but also have functions far beyond those found in biological systems. That is, ribozymes have been engineered to serve specific functions.

It is because of the ability of RNA to store information and catalyze reactions, functions normally associated with DNA and proteins, respectively, that RNA has been proposed to be the origin of life, with DNA and proteins assuming these roles after much evolution. It should also be mentioned that structural studies of RNA have, of late, been advancing more rapidly than that of protein, historically the focus of structural biology.

Other involvement of nucleic acids in enzymes involves the use of DNA as a template for new DNA synthesis. The ends of DNA strands (chromosomes) are maintained by the continuous extension of the ends. DNA is used as a template for these repetitive sequences called telomeres. Additionally, because nucleic acids readily forms duplexes, several enzymes incorporate an RNA or DNA oligonucleotide to aid in binding the protein to the DNA. Such enzymes include those required for DNA manipulation, including gene expression, DNA replication, or maintenance.

F. FORWARD-LOOKING DEVELOPMENTS

The increased use of technology has opened new avenues in molecular biology involving nucleic acids. The easy, rapid, and inexpensive synthesis of RNA and DNA has made nucleic acids a useful research tool. New methods in molecular genetic research are being developed and employed constantly.

In the construction and identification of RNAs that contain enzymatic activity, a technique known as systematic evolution of ligands by exponential enrichment (SELEX) has been used (Ref. 5). SELEX is a process of producing a small amount of a large variety of RNA sequences. These are than screened, or assayed, by their activity. Those that are found to have a chemical or biological function are then amplified by PCR and subjected to another round of functional assays. This ultimately results in the isolation of biochemically functional RNA sequences.

Thus, methods for the rapid synthesis of a variety of oligonucleotides, rather than the large-scale production of a single sequence, would be a significant advance. One such method, now being employed in the screening of expressed gene, is in situ microarray synthesis, the use of a large substratum on which a variety of oligonucleotides can be synthesized. By using a chip rather than a tube for the reaction, much smaller volumes of reagents can be used and direct targeting of reaction sites can be employed. The specifics of these methods are currently under development.

As with virtually all areas of technology, the miniaturization of these processes will allow for greater reaction parallelism and faster reaction times. For this reason, chip based synthesis and nano-scale miniaturization will allow for the screening and identification of functional oligonucleotides at a rate unattainable with current methods.

Studies still in their infancy are looking into the replacement of the ribose sugar or the nitrogenous base with various inert or functional moieties. These substitutions have been found to affect the stability, structure, and function of the resulting nucleotides, but a description of the progress and preliminary results would be incomplete and inappropriate because of the early stage of these studies.

Another variation is the protein nucleic acid (PNA). PNAs are molecules with structures resembling those of nucleic acids, but are constructed of subunits that more closely resemble those of a protein and are connected through peptide bonds. The benefits of this system are still being elucidated, but PNA/DNA duplexes and 1PNA/2DNA triplexes have been seen.

F. FUTURE ROLES OF NUCLEIC ACID TECHNOLOGIES

Gene silencing by antisense or triplex formation can be a useful method of pre-immune treatment of bacterial or viral infection. In many cases, by a time an immune response is initiated and a person become symptomatic, the infection has become too severe to treat. Antisense treatments can halt (or limit) gene expression until a successful immune response can be initiated. This would reduce the need for antibiotics and, because many antibiotic treatments preempt verification of infection, reduce the use of antibiotics as a preventive measure. Triplex technology may be used as an active treatment for such infections. In addition, triplex DNA treatment can be used against viral infections. Because, a viral infection is based on the replication of its DNA (or RNA) genome, inhibition of this process can be used as a countermeasure.

RNA and DNA molecules can be designed to bind small molecules. This process could be used as a treatment for exposure to chemical weapons. Current treatment is based on treating the symptoms and letting the chemicals be removed from the system naturally. Functional nucleic acids can provide a way to introduce an active decontamination process to the biological system. By binding the toxin, nucleic acid enzymes can render them biologically inert.

The universality of nucleotide sequences allows for the use of functional oligonucleotide multimers. Although the difference between having a single nucleotide

chain of 50 bases and two 25-base oligomers can be nominal at the functional level, it allows for functionality as a binary drug, only finding its effectiveness once both components have been introduced. It is freedom like this that often gives nucleic acids an advantage over proteins when studying the relationship between structure and function.

An improved understanding of nucleic acid structure and chemistry has provided new paths to address molecular and cellular biology. Nucleic acids are now used as a tool in biochemical research rather than being the topic of that research. The use of these tools is limited only by their availability. That is, most oligonucleotide-based protocols are based on the rapid and inexpensive synthesis of large amounts of these molecules. There is, however, an obvious shortcoming in this synthesis technology: the in vitro synthesis of small quantities of an array of nucleic acids is not possible at this time.

A library of oligonucleotides would make it easier to rapidly screen useful sequences. The SELEX method described addresses this issue, but it is different in that a library of bacteria or other expression system synthesizes the RNA in this method in vivo. The in vitro, de novo synthesis of these oligomers would again increase the availability and reduce the cost of creating such libraries. Changes to the library would be simple processes of reprogramming the computer rather than creating a new library.

In addition, in many molecular biology techniques involving oligonucleotides, a large excess of the DNA or RNA oligomers is used. This is a waste of money and reagent. If only 100 oligomers are needed, only 100 should be used. This, again, could be solved by the miniaturization of the process.

G. CONCLUSIONS

It is clear that nucleotide oligomers have found widespread use in the molecular biology laboratory and a growing interest in the clinical setting. The ongoing genome sequencing projects will bring even more uses to nucleic acid technologies. The current techniques are focused on the synthesis of large amounts of a single DNA or RNA sequence, of which very little is used, the excess being discarded. One of the major shortcomings in these technologies is the current inability to synthesize large libraries of oligomers, each in small quantity. This area has not been addressed specifically because of a limited need for such technologies as well as limited funding for an undertaking that may require investigation of new chemistries in addition to microtechnology.

The miniaturization of nucleic acid synthesis technology could increase the useful product and result in a cost decrease. In addition, miniaturization of the synthesis

apparatus would result in greater parallelization of synthesis reactions. The increased synthesis, and subsequent screening, of nucleotide oligomers will improve current technologies and enable the development of new technologies that require large amount of highly variable oligomers sequences such as in DNA computing.

GLOSSARY

A	adenosine
ADRP	Autosomal Dominant Retinitis Pigmentosa
C	cytidine
DNA	deoxyribonucleic acid
G	guanosine
mRNA	messenger RNA
OH	hydroxyl
PCR	polymerase chain reaction
PNA	protein nucleic acid
RNA	ribonucleic acid
SELEX	systematic evolution of ligands by exponential enrichment
SNP	single nucleotide polymorphism
T	thymidine
TFO	triplex forming oligo

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14. ABSTRACT

This paper describes the chemical and biological basis of nucleotide oligomers (oligonucleotides) and nucleic acids. This paper also describes (1) several uses of these molecules as tools in laboratory and clinical medical research and (2) several emerging technologies that exploit the increasing knowledge of molecular biochemistry of nucleic acids. Also addressed are the roles of nucleic acids in biological research and some emerging technologies involving these molecules.

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